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Genetic integrity of four species of *Leptidea* (Pieridae, Lepidoptera) as sampled in sympatry in West Siberia

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Abstract

In southern West Siberia, as many as four *Leptidea* Billberg, 1820 species are present sympatrically: *L. amurensis* (Ménétriés, 1859), *L. morsei* (Ménétriés, 1859), *L. sinapis* (Linnaeus, 1758) and *L. juvernica* Williams, 1946. The two latter were recently recognised as nearly sibling species on morphological and molecular characters. Specimens intermediate as to their subtle diagnostic characters occurring in West Siberia and elsewhere were interpreted as resulted from limited introgression. This supposition was tested via populational morphological and molecular analysis of spring brood specimens of all the four species taken from a limited (4.5 × 0.2 km) area in the suburbs of Novosibirsk. The samples were analysed with respect to the genitalic morphology, external characters, three nuclear (*CAD*, *H1* gene and *ITS2*) and one mitochondrial (*COI*) molecular markers, infection of the intracellular maternally inherited bacterial symbiont *Wolbachia* Hertig, 1836 and its *wsp* gene coding for a hypervariable surface protein. Interspecific variation of the nuclear *CAD* and *ITS2* sequences and the mitochondrial *COI* gene in *L. sinapis* and *L. juvernica* turned out concordant. The absence of molecular evidence of introgression suggests genetic integrity of these two species and allows their reliable identification by molecular characters. The genitalic (lengths of the saccus and valva) and external characters (wing pattern) of males overlap in *L. sinapis* and *L. juvernica,* as identified by molecular markers and thus are not so helpful in actual species identification. Only the ductus bursae length showed no overlap and can be used for identification of females. The histone H1 gene appeared five times less variable over the four studied species than *COI*, and found to be identical in species *L. sinapis* and *L. juvernica*. *Wolbachia* infection was found in all studied species. We identified three *wsp* variants of *Wolbachia*: 1) *wsp-10* allele in *L. amurensis*, *L. sinapis*, *L. juvernica*; 2) a very similar *wsp-687* allele in *L. sinapis*; and 3) *wsp-688*, highly divergent to the previous ones, in *L. morsei*.

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Keywords
Leptidea, Lepidoptera, *Wolbachia*, introgression, molecular markers, histone H1, *COI*, *ITS2*, *wsp*, genitalia morphology, intraspecific variation

Introduction

The genus *Leptidea* Billberg, 1820 (Dismorphiinae, Pieridae) includes several (at least eight) Palearctic species. Recently it attracted attention because of repeated and rather unexpected discoveries of sibling species (Dincă et al. 2011). Firstly, *Leptidea lorkovicii* Réal, 1988 was separated from the sympatric *L. sinapis* (Linnaeus, 1758) on the basis of substantial differences in the genitalia structure (Réal 1988). Later a new name *Leptidea reali* Reissinger, (1990) was proposed to it because of existence of a senior homonym *Leptidea duponcheli lorkovici* Pfeiffer, 1932 (Reissinger 1990). Note that later it was found out (Dincă et al. 2011) that the new name was not necessary and invalid because of existence of an older available name *juvernica* Williams, 1946 proposed for an Irish population showing the relevant morphology. Secondly, on the basis of molecular and karyological data, the species known under the invalid name *L. reali* was split into two allopatric species, *L. reali* s. str. from Spain, southern France and Italy (for this species the name in narrow sense is valid) and *Leptidea juvernica* Williams, 1946 ranging from the French Pyrenees in the south-west and Ireland in the north-west to Central Siberia in the east (Dincă et al. 2011). Citing literature data *de facto* dealing with *L. juvernica*, below we will use this name although before 2011 the authors used the name *L. reali*. Ranges of both *L. reali* and *L. juvernica* overlap with that of *L. sinapis* ranging from Spain and Ireland to East Siberia (Dincă et al. 2011). Hence *L. sinapis* and *L. juvernica* co-occur on a vast territory from South Europe to Central Siberia.

The main diagnostic character of *L. reali* and *L. juvernica* versus *L. sinapis* is a substantially greater relative lengths of the aedeagus and saccus (which correlate to each other) in the male genitalia and of the ductus bursae in the female genitalia (Fumi 2008, Ivonin et al. 2009, Dincă et al. 2011, Sachanowicz 2013). There are less distinct differences between *L. juvernica* and *L. sinapis* in the wing shape and pattern. For both the European part of Russia (Bolshakov 2005, Bolshakov et al. 2013) and West Siberia (Ivonin et al. 2009), the following differences between *L. juvernica* and *L. sinapis* in the wing coloration were claimed:

(i) spring brood males of *L. juvernica* have in general darker, more suffused hind wing underside below vein M3 and with less distinct stripy pattern (less expressed lighter postdiscal spots between veins) than those of *L. sinapis*;
(ii) summer brood males of *L. juvernica* differ from those of *L. sinapis* in the fore wing upperside without the light rim along the apical dark spot and darkened ends of veins M3 and Cu1;
(iii) in West Siberia, the spring brood males of *L. juvernica* were claimed to have a more attenuated fore wing apex than those of *L. sinapis* (Ivonin et al. 2009).

No external differences were revealed between females of the two species.

Authors working in different regions (Verovnik and Glogovčan 2007, Ivonin et al. 2009, Bolshakov et al. 2013) pointed out a substantial variation in these two species. Several morphs were recognised based on the tint of wing coloration, which are shared by both *L. sinapis* and *L. juvernica* (Bolshakov et al. 2013). The lengths of the aedeagus and saccus taken alone do not allow distinguishing *L. sinapis* from *L. juvernica* in all cases because of some overlap in *L. sinapis* and *L. juvernica* (Fumi 2008, Sachanowicz 2013). Tsvetkov (2007) reported that samples of *L. juvernica* from forested and open habitats differed in the average relative aedeagus length, which is less in forested habitats. It was even hypothesized that such variation could be supported by selection for a longer aedeagus to ensure mating in more windy open habitats (Bolshakov et al. 2013); however, this would demand a long lasting maintenance of genetic isolation between the two habitat types, which hardly exists at all. Bolshakov et al. (2013) reported specimens with intermediate genitalia from Mordovia Republic (European Russia), e.g. males of *L. juvernica* with a normally long aedeagus but the saccus short as in *sinapis* and curved as in *juvernica*. Ivonin et al. (2009) reported the occurrence in Novosibirsk Province (West Siberia, Russia) of external characters of *L. juvernica* among males with the genitalia of *L. sinapis* (with a short and straight saccus) but not vice versa, for no external characters of *L. sinapis* were found in males with the genitalia of *L. juvernica* (with a long and S-like curved saccus). Verovnik and Glogovčan (2007) reported the occurrence of males that were intermediate between *L. sinapis* and *L. juvernica* in Slovenia, namely: (i) with the aedeagus of intermediate length; (ii) with long saccus but short aedeagus and (iii) with the genitalia of *L. juvernica* but closer to *L. sinapis* according to RAPD markers.

These facts can be interpreted in three ways: (i) as resulting from some gene exchange (introgression) between *L. sinapis* and *L. juvernica*; (ii) as common polymorphism of genes affecting the genitalia structure and/or wing coloration, inherited from the common ancestor, or (iii) by independent mutations (homoplasy) of these hypothetical genes.

The relationships of closely related species may be clarified via two approaches, the phylogeographic and population genetic ones. The former approach implies accumulation of data from a territory as broad as possible in order to reconstruct the history of divergence and spread of species. The latter approach consists of analysing large samples from certain populations in order to register phenomena such as deviations from panmixia, linkage disequilibrium, gene exchange between sympatric taxa, and effects of natural selection.

Relationships between sibling species of *Leptidea* were mostly studied via the phylogeographic approach applied to the entire species ranges (Lukhtanov et al. 2011, Dincă et al. 2011, 2013) using the mitochondrial *COI* and *ND1* genes, the nuclear *CAD*, *ITS2*, and *Wg* markers, and karyotype. These studies did not reveal any introgression between *L. reali*, *L. juvernica* and *L. sinapis* (Lukhtanov et al. 2011, Dincă et al. 2011, 2013). Examples of population genetic studies are the analysis of several sympatric populations of *L. reali* and *L. sinapis* in the French Pyrenees (Martin et al. 2003) and of *L. juvernica* and *L. sinapis* in Slovenia (Verovnik and

Glogovčan 2007). Martin et al. (2003) rejected the introgression hypothesis while Verovnik and Glogovčan (2007) did not exclude some degree of gene exchange between *L. sinapis* and *L. juvernica*.

Another approach is searching for particular mechanisms of isolation between these two species. Hybridisation experiments revealed that prezygotic isolation between *L. sinapis* and *L. juvernica* or *L. reali* was probably based on behavioral barriers, for instance recognition by females of a species-specific courtship behaviour of males or species-specific pheromones (Friberg et al. 2008b, Dincă et al. 2013). Non-conspecific matings between these species were not observed while conspecific individuals from geographically remote populations mated successfully (Dincă et al. 2013). A shifted flight period and some habitat segregation also contribute to the prezygotic isolation between *L. sinapis* and *L. juvernica* (Friberg et al. 2008a). Differences in larval foodplant species were not found (Friberg and Wiklund 2009).

The western foothills of the Altay-Sayan Mountain System (West Siberia, Russia) are unique in being inhabited by four *Leptidea* species altogether, more than elsewhere in the world: *Leptidea morsei* (Fenton, 1881), *L. amurensis* (Ménétriés, 1859), *L. sinapis* and *L. juvernica* (Fig. 1). They have different habitat preferences: at least in Novosibirsk Province: *L. morsei* mostly inhabits open woods, *L. sinapis* — various meadows, *L. amurensis* and *L. juvernica* mostly inhabit meadow steppes in rough relief terrains (Ivonin et al. 2009). In spite of these preferences, all the four species coexist with nearly equal abundance and similar flight period on grassy glades on the eastern bank of the Novosibirsk Water Reserve in the Novosibirsk Academy Town (Kosterin et al. 2007), making this territory an excellent site for studying isolation *vs* introgression of *Leptidea* spp. Hence, we attempted a pure population genetic approach and analysed a sample of spring brood specimens of *Leptidea* collected from the same small area at the junction of the Novosibirsk city and Berdsk town.

The main attention was paid to the closely related and supposedly hybridising species *L. sinapis* and *L. juvernica*. They were analysed with respect to the popular mitochondrial marker *COI* (the gene for cytochrome oxidase I) and the nuclear markers *CAD* (the gene for carbamoyl phosphate synthetase II, aspartate carbamoyltransferase, dihydroorotase), *ITS2* (internal transcribed spacer 2 in the ribosome cluster), and the histone H1 gene, designated here as *H1*. A histone H1 gene was recently proposed as a good phylogenetic marker (Zaytseva et al. 2012, 2015, Solovyev et al. 2015). We also analysed infection by the maternally inherited endosymbiont *Wolbachia* Hertig, 1836 (Zhou et al. 1998; Van Meer et al. 1999; Baldo and Werren 2007), and sequences of its highly variable gene *wsp* (*Wolbachia* surface protein). In addition, the males of *L. sinapis* and *L. juvernica* were analysed for the lengths of the valve and saccus in the male genitalia and scored for the wing characters, and the ductus bursae was measured in females. The *COI*, histone *H1* and *wsp* genes were also sequenced and the *Wolbachia* infection was assessed in the two other co-occurring species, *L. morsei* and *L. amurensis*. Since their external characters are constant and sufficient for reliable identification, their genitalia were not examined.

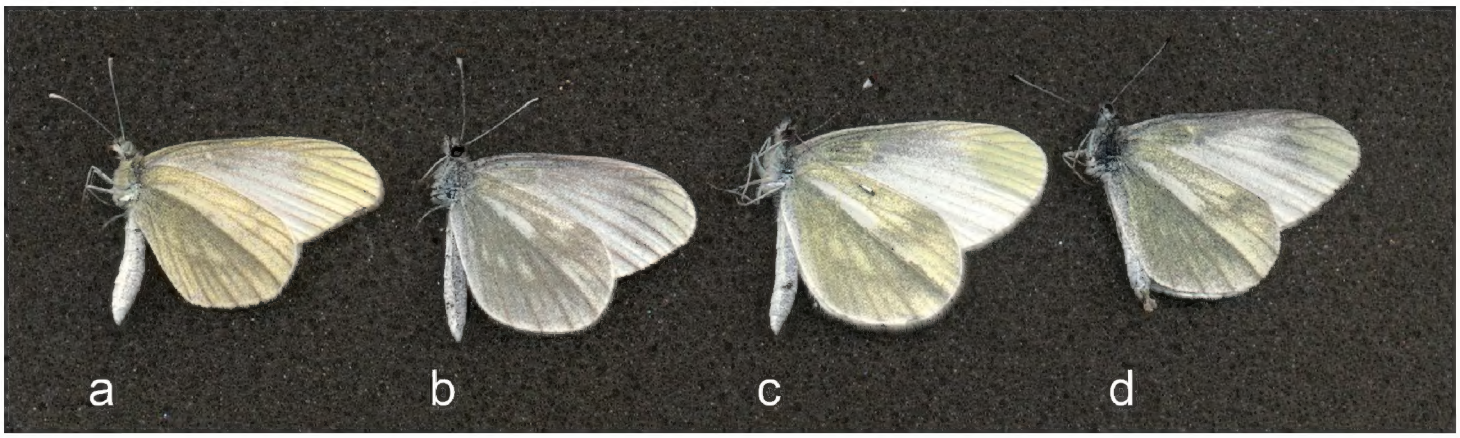


Figure 1. Spring brood males of four species of *Leptidea* Billberg, 1820: *L. amurensis* (Ménétriés, 1859) (**a**), *L. morsei* (Fenton, 1881) (**b**), *L. sinapis* (Linnaeus, 1758) (**c**) and *L. juvernica* Williams, 1946 (**d**), simultaneously collected in the studied area at the border of Novosibirsk city and Berdsk town, West Siberia, Russia (after Ivonin et al. 2009). Note the difference in the shape of the fore wing apex.

Materials and methods

Material

Seventy spring brood specimens of *Leptidea* spp. were collected in the vicinity of Novosibirsk Academy Town, Novosibirsk Province. The collection area was a 100–200 m wide and a 4.5 km long continuous stripe of meadows adjacent to birch/ pine forests, along the bank of the Novosibirsk Water Reserve and the parallel railroad, between Obskoe More railway station (54°47'37"N; 83°04'34"E; (DMS)) and a point (54°50'04"N; 83°04'40"E (DMS)), 900 m NNE of Rechkunovka railway station, at elevations of 107–137 m a.s.l. (see the locality on a schematic map of northern Eurasia in Fig. 2). Note that the northern half of this area belongs to the city of Novosibirsk while the southern half to the satellite town of Berdsk (with the border at Beregovaya railway station in the middle of the collecting area). Berdsk is the type locality of the subspecies *Leptidea reali yakovlevi* Mazel, 2003 (the justification of which to our opinion have been insufficiently reasoned in the original description). The specimens were collected by O.E. Kosterin in June 2010, May 2011 and May 2012 with a net and frozen immediately. Details of the specimens examined are provided in Table 1. In screening for *Wolbachia* infection, the combined sample of *L. juvernica* and *L. sinapis* was updated with 15 more specimens which were not identified to either of these two species, not analysed in other respects and not included into Table 1, so that the total sample of *Wolbachia* screening contained 85 *Leptidea* specimens.

DNA extraction

Genomic DNA was extracted according to Bogdanova et al. (2009), with modifications for isolation from individual insects. Frozen specimens without abdomen and wings were homogenized in 0.6 ml 0.15 M NaCl. The homogenate was centrifuged

Table 1. Material collected, *COI* gene allelic states as revealed by CAPS approach (denoted as follows: s – *Leptidea sinapis*, j – *L. juvernica*, a – *L. amurensis*, m – *L. morsei*), European Nucleotide Archive (ENA) accession numbers of the *COI* and *H1* gene sequences, presence of *Wolbachia* infection (+ detected; - not detected) and the *wsp* alleles according to the PubMLST database.

Specimen	Sex	Date collection	*COI* variant	*COI* ENA accession number	*H1* ENA accession number	*Wolbachia* infection (*wsp* allele)
L1	♂	05.06.2010	s			-
L2	♂	05.06.2010	j			+
L3	♂	05.06.2010	j			+
L4	♂	05.06.2010	s			+
L5	♂	05.06.2010	j			+
L6	♂	05.06.2010	s			+
L7	♂	06.06.2010	s			+
L8	♂	06.06.2010	j			+
L9	♂	06.06.2010	j			+
L10	♂	05.06.2010	j	HG969218	LN606440	+
L11	♂	29.05.2011	j	HG969219		+
L12	♂	29.05.2011	j	HG969220	LN606441	+ (*wsp*-10)
L13	♂	29.05.2011	j	HG969221		+
L14	♂	29.05.2011	j	HG969222		+
L15	♂	29.05.2011	s	HG969223	LN606442	+
L16	♂	29.05.2011	s	HG969224	LN606443	+ (*wsp*-687)
L17	♂	29.05.2011	s	HG969225	LN606444	+ (*wsp*-10)
L18	♂	29.05.2011	s	HG969226		+
L19	♂	13.05.2012	j	HG969227		+
L20	♂	13.05.2012	j			+
L21	♂	13.05.2012	j			+
L22	♂	13.05.2012	s			+
L23	♂	13.05.2012	s			+
L24	♂	14.05.2012	j			+
L25	♂	14.05.2012	s			+
L26	♂	15.05.2012	s			-
L27	♂	15.05.2012	j			+
L28	♂	15.05.2012	j			+
L29	♀	29.05.2010	j			+
L30	♀	05.06.2010	j			+
L31	♀	05.06.2010	j			+
L32	♀	05.06.2010	j			+
L33	♀	05.06.2010	j			+

Specimen	Sex	Date collection	*COI* variant	*COI* ENA accession number	*H1* ENA accession number	*Wolbachia* infection (*wsp* allele)
L34	♀	05.06.2010	s			-
L35	♀	05.06.2010	j			+
L36	♀	06.06.2010	j			+
L37	♀	06.06.2010	s			+
L38	♀	06.06.2010	j			+
L39	♀	13.05.2012	j			+
L40	♀	13.05.2012	j			+
L41	♀	13.05.2012	j			+
L42	♀	13.05.2012	j			+
L43	♀	14.05.2012	s			+
L44	♀	14.05.2012	s			+
L45	♀	14.05.2012	j			+
L46	♀	14.05.2012	s			+
L47	♂	29.05.2010	a	HG969228		+
L48	♂	29.05.2010	a	HG969229		+
L49	♂	26.05.2011	a	HG969230		+
L50	♂	26.05.2011	a	HG969231	LN606445	+
L51	♂	29.05.2011	a	HG969232		+
L52	♂	29.05.2011	a	HG969233		+
L53	♂	29.05.2011	a	HG969234		+
L54	♀	28.05.2010	a	HG969235		+
L55	♀	28.05.2010	a	HG969236		+
L56	♀	26.05.2011	a	HG969237		+
L57	♀	26.05.2011	a	HG969238		+
L58	♀	26.05.2011	a	HG969239	LN606446	+ (*wsp*-10)
L59	♀	29.05.2011	a	HG969240		+
L60	♂	29.05.2010	m	HG969241	LN606447	+
L61	♂	29.05.2011	m	HG969242		+
L62	♂	29.05.2011	m	HG969243		+
L63	♂	29.05.2011	m	HG969244		+
L64	♂	29.05.2011	m	HG969245		+
L65	♂	29.05.2011	m	HG969246		+
L66	♀	29.05.2010	m	HG969247		+
L67	♀	26.05.2011	m	HG969248	LN606448	+ (*wsp*-686)
L68	♀	29.05.2011	m	HG969249		+
L69	♀	29.05.2011	m	HG969250		+
L70	♀	29.05.2011	m	HG969251		+

Figure 2. Position (black circle) of the studied area at the border of Novosibirsk city and Berdsk town (54°47'37"N; 83°04'34"E – 54°50'04"N; 83°04'40"E; DMS), Novosibirsk Province, Russia, on a schematic map of northern Eurasia.

(3,300 rcf, 5 min) and the supernatant was discarded, then 0.2 ml solution for DNA extraction (0.1 Tris-HCl, pH 8.0; 5 mM EDTA; 0.5% SDS; 0.1 M NaCl) was added and incubated at room temperature for 40 minutes. Then the solution was centrifuged (16,100 rcf, 5 min) and the pellet was discarded. To remove proteins and RNA, LiCl (0.2 ml, 5M) was added to the supernatant solution and incubated on ice for 15 min. The solution was centrifuged (16,100 rcf, 5 min) and the supernatant was transferred to fresh tubes. Ethanol (1 ml, 96%) was added and the mixture was incubated on ice for an hour. Then it was centrifuged (16,100 rcf, 10 min) and the supernatant was discarded. The precipitate was washed with 0.1 ml 75% ethanol and centrifuged (16,100 rcf, 5 min), then dried at 50 °C for 5 min and dissolved in 50 ⊠l of deionized H_2O.

DNA amplification and sequencing

A 708 bp long fragment of the *COI* gene, positions 1526–2156 (positions are given according to the mitochondrial reference of *Drosophila yakuba* Burla, 1954 (AN X03240)), was amplified with the universal insect primers LCO-1490 and HCO-2198 (Folmer et al. 1994). A 684 bp sequence of *ITS2* (internal transcribed spacer 2) and 571 bp sequence of *CAD* (carbamoyl phosphate synthase II, Aspartate carbamoyltransferase, dihydroorotase) were amplified with primer pairs ITS3/ITS4 and CADFa/CADRa, respectively, following Dincă et al. (2011). The *H1* gene was amplified with

primers designed for two overlapping sequences: the 5' terminal part with the primer pair LH4-f (5'ACCCTGTACGGTTTCGGCGGTTAA) and HeH1-r (5' AGCGCCCTTGCCCTTGGTCTGTATC) and the 3'terminal part of gene with another pair, HeH1-f (5'ACCCACCCCAAGACCTCCGAGATGGT) and LeH1C-r (5'AGGGGGACTCACTTTTTGGA). The 5' terminal fragment is approximately 1.5 kbp long, and the 3'-terminal fragment is 650 bp long. The primers were originally designed to match orthologous sequences of *Bombyx mori* (Linnaeus, 1758) (LH4-f), *Heliconius erato* (Linnaeus, 1758) (HeH1-r and HeH1-f) (Solovyev et al. 2015) and *L. sinapis* (LeH1C-r); they were produced by Biosset (Novosibirsk. Russia).

DNA samples were examined for *Wolbachia* infection by amplification of *wsp* with the following primer set: wsp81F (5'TGGTCCAATAAGTGATGAAGAAAC-3'), wsp691R (5'AAAAATTAAACGCTACTCCA-3') (Braig et al. 1998). PCR products of five DNA stocks of four species were sequenced.

PCR mixtures (30 ☒l) contained 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 25mM KCl, 60 mM Tris-HCl (pH 8.5), 10 mM β-mercaptoethanol, 0.1% Triton X-100, 0.5 μM of each primer, 1 ☒l of genomic DNA solution and 1 U of Taq DNA polymerase or 1 U of Smart-Taq DNA Polymerase (by Laboratory Medigen, Novosibirsk, Russia). PCR was performed using a thermal cycler MyCycler (Bio-Rad, USA) with the following program: 1) 94 °C — 2 min 30 s, 1 cycle; 2) 95 °C — 15 s, 47–55 °C — 30 s, 68 °C — 1 min, 35 cycles; 3) 68 °C — 2 min, 1 cycle.

The entire coding sequence of *H1* and a 631 bp long fragment of *COI* (positions 1526–2156) were sequenced. The Sanger reaction was conducted in 30 ☒l volume of mixture containing 1 ☒l of BigDye Terminator, version 3.1 (Applied Biosystems), 100–200 ng of DNA, 3 pmol of primer and 6 ☒l of buffer solution for BigDye 3.1. A MyCycler (Biorad) thermocycler was used with the following program: 95 °C — 45 s, 50 °C — 30 s, 60 °C — 4 min; 26 cycles. Sequencing was made at the SB RAS Genomic Core Facility, Novosibirsk.

Sequence alignments and calculation of the genetic distances were performed using the MEGA 5.0 software package (Tamura et al. 2011).

CAPS genotyping *L. sinapis* and *L. juvernica*

For genotyping the *L. sinapis* and *L. juvernica* specimens with respect to certain diagnostic nucleotide substitutions in mitochondrial and nuclear markers, CAPS analysis was conducted (Konieczny and Ausubel 1993). After the analysis of DNA sequences of *L. sinapis* and *L. juvernica* in public databases, we picked the set of endonucleases *Hpa*II, *Alu*I and *Hind*III for genotyping the *COI* gene, *ITS2* region and *CAD* gene, respectively.

The 708 bp long amplified fragment of *COI* of *L. juvernica* contains three restriction sites for endonuclease *Hpa*II and is digested to 4 fragments (66, 109, 206, 327 bp), while the orthologous fragment of *L. sinapis* has no restriction sites. The *ITS2* region of *L. sinapis* contains the only site specific for endonuclease *Alu*I and is digested into 2 fragments (412, 272 bp); the *ITS2* of *L. juvernica* does not contain restriction

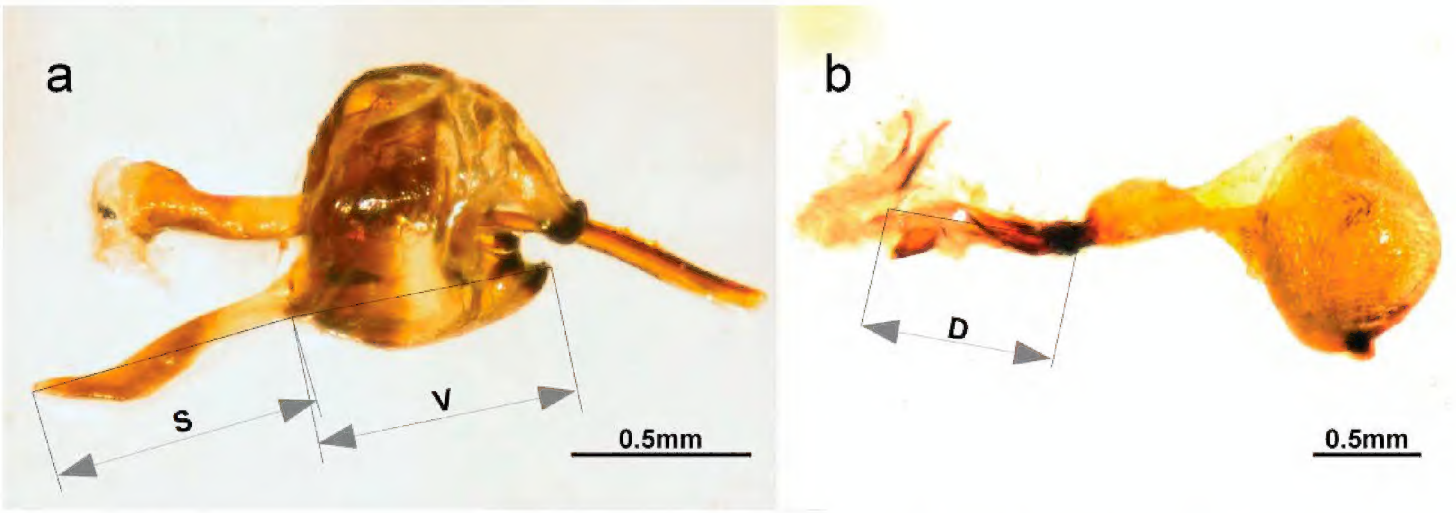


Figure 3. Male (**a** specimen L12) and female (**b** specimen L45) genitalia of *Leptidea juvernica*, with measured parameters indicated, namely the length of the saccus (S), valve (V) and ductus bursae (D).

sites for *Alu*I. The *CAD* sequence of *L. juvernica* includes only one restriction site for endonuclease *Hind*III, which digests it into two fragments, 110 and 461 bp in length; the *CAD* sequence of *L. sinapis* has two sites which produce three digestion fragments (110, 189, 272 bp). The buffers and enzymes for restriction reactions were produced by Sibenzim, Novosibirsk, Russia. The identical procedure was used for different markers, as follows: 9 ⊠l of the PCR product was added with 0.5 U of endonuclease and 1 ⊠l of a buffer relevant to the endonuclease. The mixture was incubated at 37 °C for 2 hr, inactivated at 80 °C for 20 min and analyzed by electrophoresis in 1.5% agarose.

Genitalia morphometrics

The abdomen tip with the genitalia was taken from frozen specimens of *L. juvernica* and *L. sinapis*, incubated for 10 min at 98 °C in 10% potassium hydroxide for maceration and dissected under a stereomicroscope. Lengths of the valve (V) and saccus (S) were measured with an ocular-micrometer and binocular lens MBS-2, as shown in Fig. 3. Besides, the saccus curvature was classified as referring to arbitrary binary scores: 0 – straight, 1 – S-like curved.

Statistical analyses were carried out using MS Excel 10 for Windows.

The genitalia were analysed before molecular analysis, which was carried out blindly of the genitalic results. The specimens in which molecular results appeared discordant with morphological ones, were then rechecked for morphology and discordancy was confirmed.

External characters

The external characters reported to be different in the spring brood males of *L. juvernica* and *L. sinapis*, namely (1) the wing underside below vein M3 more suffused by dark scales and with less expressed lighter spots between veins and (ii) more attenuate fore

Figure 4. Males of *Leptidea sinapis* (**a** specimen L1 **b** specimen L26) and *Leptidea juvernica*, (**c** specimen L3 **d** specimen L8), with different scores of subjectively evaluated wing characters: the hind wing underside suffusion below vein M3: 0 – lighter, with better expressed lighter spots between veins; 1 – stronger, with scarcely seen lighter spots; and the fore wing apex shape: 1 – more acute, 0 – more rounded. The scores for the shown specimens are as follows (suffusion, apex shape): **a** (0,0); **b** (1,0); **c** (1,1); **d** (1,0); **a** and **c** are variants most frequent in the respective species.

wing apex in the former species (Ivonin et al. 2009), are difficult to measure and somewhat subjective. Therefore we classified them as referring to arbitrary binary classes:

- hind wing underside suffusion below vein M3: 0 – with well-expressed lighter spots between veins, 1 – stronger, more even, with scarcely or expressed lighter spots;
- fore wing apex: 0 – broadly rounded; 1 – more attenuated and acute (Fig. 4).

Results

Inter- and intraspecies variation of mitochondrial *COI* gene

The 631 bp long fragment of the mitochondrial gene *COI* (position 1526 – 2156) was sequenced for 34 *Leptidea* specimens (10 of *L. sinapis* + *L. juvernica*, 11 of *L.*

Table 2. Polymorphic positions in the *COI* gene in *Leptidea sinapis* (specimens L15-L18) and *L. juvernica* (specimens L10–L14 and L19). Positions discriminating *s*- and *j*- allele types are boldfaced; intraspecific substitutions are underlined.

Specimens representing six alleles		L10, L11, L14	L12, L13	L19	L15	L16, L17	L18
allele type		**j**	**j**	**j**	**s**	**s**	**s**
Position	**1530**	**T**	**T**	**T**	**C**	**C**	**C**
	1587	A	A	A	A	G	G
	1599	**C**	**C**	**C**	**T**	**T**	**T**
	1615	A	G	G	G	G	G
	1624	**A**	**A**	**A**	**G**	**G**	**G**
	1659	**T**	**T**	**T**	**C**	**C**	**C**
	1674	G	G	G	G	A	A
	1686	T	C	C	C	C	C
	1720	**C**	**C**	**C**	**T**	**T**	**T**
	1854	**C**	**C**	**C**	**T**	**T**	**T**
	1860	**A**	**A**	**A**	**G**	**G**	**G**
	1914	**T**	**T**	**T**	**C**	**C**	**C**
	1917	C	C	T	T	T	T
	1926	**C**	**C**	**C**	**T**	**T**	**T**
	1947	**A**	**A**	**A**	**G**	**G**	**G**
	1959	**C**	**C**	**C**	**T**	**T**	**T**
	2076	T	T	T	T	T	A
	2103	**C**	**C**	**C**	**T**	**T**	**T**
	2121	**T**	**T**	**T**	**A**	**A**	**A**
	2133	**G**	**G**	**G**	**A**	**A**	**A**
	2148	**C**	**C**	**C**	**T**	**T**	**T**

morsei and 13 of *L. amurensis*) collected in the same locality. The sequences were submitted to European Nucleotide Archive (ENA), for accession numbers see Table 1. The sequences of *L. morsei* specimens were identical. In *L. amurensis*, two alleles were found which differed in position 1969, occupied by either T or A. The 1969T allele was found in 12 specimens while the 1969A allele was only found in only specimen L57. Six *COI* alleles were revealed in *L. sinapis* and *L. juvernica*. These six alleles differed in 22 sites (Table 2) and formed two groups of three alleles each, further referred to as the *s*- and *j*-alleles. The consensuses of each group differed in 17 substitutions, the other 5 substitutions were not diagnostic. The *j*-alleles differed from each other in substitutions in the positions 1615, 1686, 1917. Two of the *s*-alleles differed in T/A substitution in position 2076, while the third, found in specimen L15, has positions 1587 and 1674 occupied by the nucleotides otherwise specific for *j*-alleles. Hence the two latter positions were not diagnostic. As a result, the set of positions diagnostic for the *s*- and *j*-type, which allows species identification, included 15 positions (Table 2).

Table 3. Evolutionary Divergence over Sequence Pairs between *Leptidea* species in the studied sample as calculated from 34 *COI* gene sequences obtained. The number of nucleotide substitutions per site averaged over all possible specimens pairs for any two species, ± its standard error, is shown below the main diagonal, their minimum value among all specimens pairs for any two species is shown above the main diagonal. The total number of positions was 631.

	1	2	3	4
1. *L. juvernica*		0.024	0.041	0.052
2. *L. sinapis*	0.029±0.006		0.043	0.052
3. *L. morsei*	0.043±0.008	0.043±0.008		0.048
4. *L. amurensis*	0.055±0.008	0.053±0.008	0.048±0.008	

The averaged and minimum *p*-distances between of the studied *COI* fragment between *L. juvernica* (*j*-alleles) , *L. sinapis* (*s*-alleles) *L. morsei*, *L. amurensis*, of are provided in Table 3.

The *wsp* gene variation

PCR amplification of the *wsp* gene revealed *Wolbachia* infection in 38 of 42 tested males and 18 of 19 tested females of the *L. sinapis* + *L. juvernica* united sample (91.8% prevalence), in all 11 tested specimens of *L. morsei* and in all 13 tested specimens of *L. amurensis* (100% prevalence) (Table 1, but 15 specimens of *L. sinapis* or *L. juvernica*, analysed only for *wsp*, are not included into the table).

The *wsp* gene was sequenced in one specimen of each *L. amurensis* (L58), *L. morsei* (L67) and *L. juvernica* (L12) and two specimens of *L. sinapis* (L16, L17). The sequences were submitted to the PubMLST database http://pubmlst.org [accessed 30 January 2015] (for accession numbers see Table 1). *L. amurensis*, *L. juvernica* and one specimen (L17) of *L. sinapis* turned out to have allele *wsp*-10, with the following hypervariable regions: *HVR1-10*, *HVR2-8*, *HVR3-10*, *HVR4-8*. The specimen L16 of *L. sinapis* had *wsp-687* allele, which differed from *wsp*-10 with one non-synonymous nucleotide substitution A193G (serine to glycine). This allele had not been previously recorded and was designated at http://pubmlst.org as *wsp-687*. *L. morsei* had a *Wolbachia* strain with another new allele, designated as *wsp*-688. This allele differed from *wsp-10* with 81 nucleotide substitutions (uncorrected *p*-distance 0.169) and gaps, resulting in 41 amino acid differences, and had the following hypervariable regions: *HVR1-2*, *HVR2-267*, *HVR3-2*, *HVR4-23*.

Concordance of mitochondrial and nuclear markers in *Leptidea sinapis* vs *L. juvernica*

The CAPS approach (see 'Materials and methods') allowed us to test 36 more specimens of *L. sinapis*/*L. juvernica* in addition to those 10 in which *COI* was sequenced.

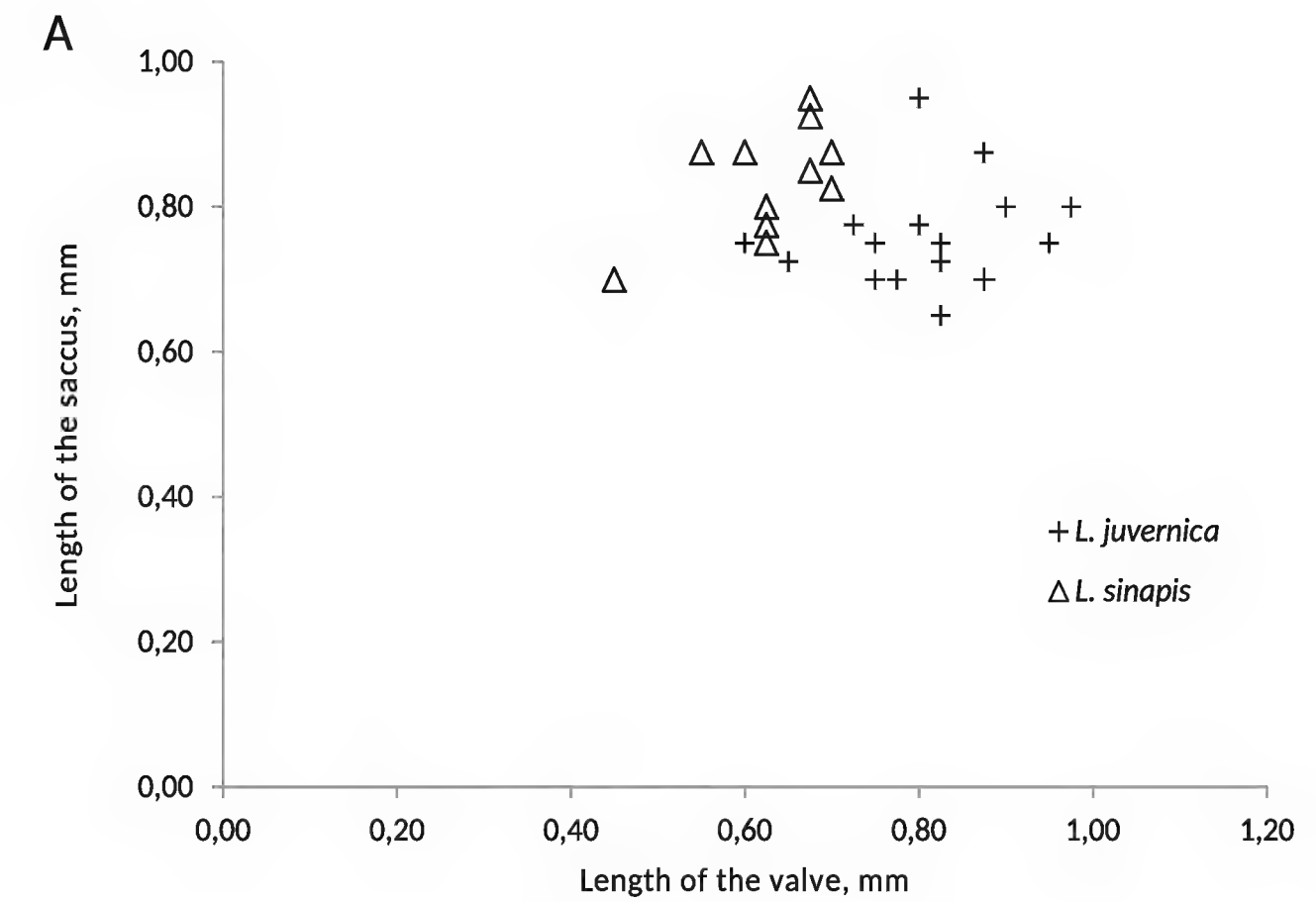
We distinguished *s*- versus *j*-alleles of the mitochondrial marker *COI* and nuclear markers *CAD* and *ITS2* in the same set of specimens. The three sets of CAPS data, for all three markers, were fully concordant: each specimen possessed either only *s*- or only *j*-alleles for all three markers. This gave us a reason to consider and further refer these specimens as belonging to the true biological species *L. sinapis* and *L. juvernica*, respectively.

The 747 bp long coding sequence of the *H1* gene of histone H1 was sequenced in 9 specimens: L10, L12 (*L. juvernica*), L15–L17 (*L. sinapis*), L50, L58 (*L. amurensis*), L60, L67 (*L. morsei*); the sequences were submitted to ENA (for the accession numbers see Table 1). *L. sinapis* and *L. juvernica* appeared to have identical primary structure of the *H1* coding sequence. Comparison of those of *L. sinapis*, *L. juvernica*, *L. morsei* and *L. amurensis* revealed 10 polymorphic sites, seven of which reside in the region coding for the C-terminal domain. As compared to the consensus *H1* coding sequence for all the four species, the *H1* sequence of *L. morsei* has two transitions, G570A and A654G, while that of both *L. sinapis* and *L. juvernica* has two transitions, G27A and G63A, and two transversions, C456G and A648C. *H1* of *L. amurensis* has three transitions G36A, G346A, G456A, and 1 transversion C453G. The substitution G346A was in the codon first position and lead to the amino acid substitution A116T, while all other above mentioned substitutions are in the third positions and synonymous. Besides, the sequenograms of both studied specimens of *L. amurensis* showed in position 306 overlapping peaks for G (as in the consensus) and T (synonymous substitutions). In one of those specimens (L50), analogous simultaneous presence of both C and T was revealed in position 219. This could result from either heterozygosity for two alleles in homologous histone gene clusters and/or cis-heterogeneity for the repeated *H1* copies in the same cluster.

Correlation of molecular markers and morphological characters in the group *L. sinapis* + *L. juvernica*

The lengths of the following genital structures were measured: the saccus and valve in males (Tables 4 and 5) and the ductus bursae in females (Tables 4 and 6). Besides, in males, we qualitatively evaluated additional characters such as the shape of saccus (straight versus S-like curved) and some wing characters (Table 7). Females of these species did not differ in external characters.

Two classes of spring brood females of the *s*- and *j*-groups with respect to the ductus bursae length were concordant with the CAPS data. The mean ductus length was significantly ($p<.001$) inferior in the s-group, and the length distributions of these groups did not overlap (Tables 4 and 6). In males, the difference between groups in the mean lengths of the saccus and valve were significant as well, with $p<.001$ and $p<.01$, respectively (Table 4). At the same time, the distributions of both the saccus and valve lengths of the *s*- and *j*-groups overlapped (Fig. 5).



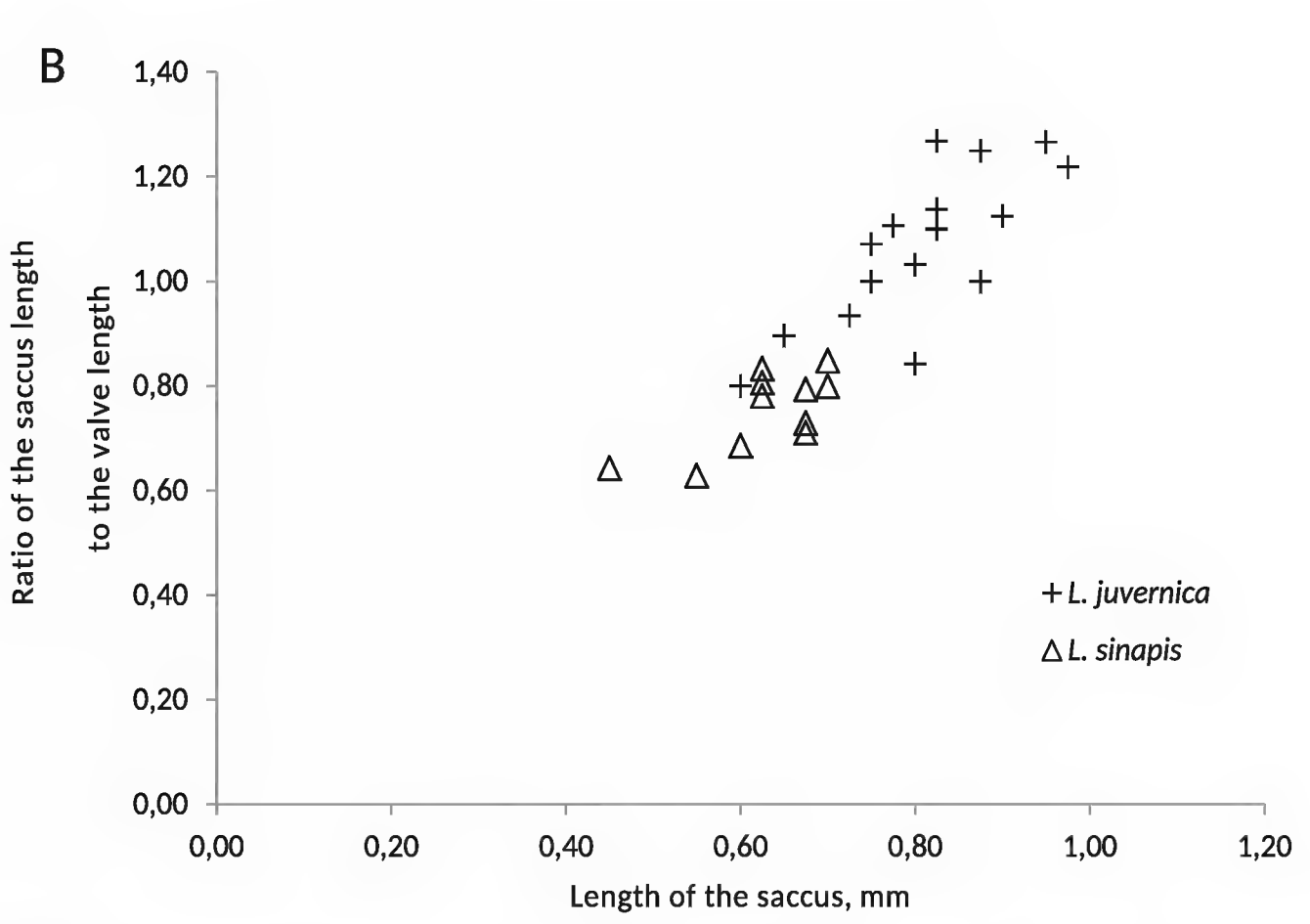


Figure 5. The saccus and valve lengths of the *L. sinapis* and *L. juvernica*. **A** Plot of the saccus length against the valve length of *L. sinapis* and *L. juvernica*, as identified by molecular markers **B** Plot of the ratio of the saccus length to the valve length against the saccus length for the same sample.

Table 4. The genital measurements of the studied samples of *Leptidea* spp. The mean values and standard deviations are given of the lengths for the saccus (*S*), valve (*V*) and their ratio (*S*/*V*) in the male genitalia and the length of the ductus bursae (*D*) in the female genitalia in the studied samples of *Leptidea sinapis* and *L. juvernica*, as identified by molecular markers, and the united sample of both species.

Parameter	Sample	*S* mm	*V* mm	*S*/*V*	*D* mm
mean	*L. juvernica*	0.81	0.76	1.07	0.96
	L. sinapis	0.63	0.84	0.75	0.58
	both species	0.73	0.79	0.93	0.85
standard deviation	*L. juvernica*	0.10	0.07	0.15	0.16
	L. sinapis	0.07	0.07	0.07	0.03
	both species	0.13	0.08	0.20	0.22
T-criterion for differentiation between the species		5.60	2.84	7.49	9.42
significance		P < 0.001	P < 0.01	P < 0.001	P < 0.001

Table 5. CAPS-analysis data, the lengths of the saccus (*S*), valve (*V*) and their ratio (*S*/*V*) in males of *L. sinapis* and *L. juvernica*.

Specimen	CAPS-analysis data (gene/restriction endonuclease)			Measurements		
	COII/ *Hpa*II	*ITS2*/ *Alu*I	*CAD* *Hind*III	*S* mm	*V* mm	ratio *S*/*V*
L1	s	s	s	0.60	0.88	0.69
L2	j	j	j	0.83	0.73	1.14
L3	j	j	j	0.80	0.78	1.03
L4	s	s	s	0.63	0.80	0.78
L5	j	j	j	0.78	0.70	1.11
L6	s	s	s	0.45	0.70	0.64
L7	s	s	s	0.60	0.88	0.69
L8	j	j	j	0.88	0.70	1.25
L9	j	j	j	0.75	0.70	1.07
L10	j	j	j	0.60	0.75	0.80
L11	j	j	j	0.73	0.78	0.94
L12	j	j	j	0.75	0.75	1.00
L13	j	j	j	0.83	0.75	1.10
L14	j	j	j	0.90	0.80	1.13
L15	s	s	s	0.70	0.88	0.80
L16	s	s	s	0.55	0.88	0.63
L17	s	s	s	0.68	0.95	0.71
L18	s	s	s	0.68	0.93	0.73
L19	j	j	j	0.65	0.73	0.90
L20	j	j	j	0.95	0.75	1.27
L21	j	j	j	0.83	0.65	1.27
L22	s	s	s	0.68	0.85	0.79
L23	s	s	s	0.63	0.78	0.81
L24	j	j	j	0.80	0.95	0.84
L25	s	s	s	0.70	0.83	0.85
L26	s	s	s	0.63	0.75	0.83
L27	j	j	j	0.88	0.88	1.00
L28	j	j	j	0.98	0.80	1.22

Table 6. CAPS-analysis data and the lengths of the ductus in females of *L. sinapis* and *L. juvernica*.

Specimen	CAPS-analysis data (gene/restriction endonuclease)			The length of ductus (mm)
	COI/ *Hpa*II	*ITS2*/ *Alu*I	*CAD*/ *Hind*III	
L29	j	j	j	1.00
L30	j	j	j	1.10
L31	j	j	j	0.83
L32	j	j	j	0.78
L33	j	j	j	0.95
L34	s	s	s	0.60
L35	j	j	j	1.00
L36	j	j	j	0.95
L37	s	s	s	0.60
L38	j	j	j	0.88
L39	j	j	j	0.85
L40	j	j	j	1.23
L41	j	j	j	0.75
L42	j	j	j	1.25
L43	s	s	s	0.55
L44	s	s	s	0.55
L45	j	j	j	0.90
L46	s	s	s	0.60

The saccus curvature did not appear as a reliable differentiating character as well, since its mean square contingency coefficient (φ coefficient) value with the CAPS data was rather small ($\varphi = 0.50$, $p<.001$).

The size, coloration of the hind wing underside and the shape of apex of the fore wing were also found to associate with the molecular groups *j* and *s* but again with small values of the φ coefficient: $\varphi = 0.49$ for the size, $\varphi = 0.73$ for the hind wing coloration $p<0.001$; $\varphi = 0.29$ for the fore wing apex $p<0.05$.

It may be concluded that neither the genital structure lengths, nor the saccus curvature, nor the general size, nor the wing coloration allow reliable identification of males of the s- and j-groups.

Discussion

Genetic integrity of the species *L. sinapis* and *L. juvernica* in the studied location

The observed differences in the studied *COI* fragment of *L. sinapis* and *L. juvernica* are substantial. They are illustrated by the averaged and minimum p-distances provided in Table 3 (both values being very close to each other). This result well agrees with the earlier published data (Dincă et al. 2011, 2013, Lukhtanov et al. 2011). Full concordance of alleles of two unlinked nuclear genes and mitochondrial genes unequivocally supports the

existence of integrated "molecular species" which can be identified based on diagnostic nucleotide substitutions in either of the mentioned genes, by CAPS-analysis or direct sequencing. Since any interspecies cross would bring about discordance of these markers, that we did not detect, the gene flow between these 'molecular species' is either absent or very limited. Thus, our 'molecular species' are at the same time valid biological species according to the Mayerian species concept. They are to be identified as taxonomical species *Leptidea sinapis* and *L. juvernica*, according to the predominating morphological characters used to be considered diagnostic for species bearing these names. At the same time, we claim that these morphological characters are not satisfactory for species identification, since opposite variants of each of them are still present in each of the two species.

Insufficiency of morphological and colorational characters for identification of the species *L. sinapis* and *L. juvernica*

In some studies the task of quick and still reliable identification of species of the *L. sinapis* complex by application of a morphometric approach was achieved with a 100% efficiency (Fumi 2008, Sachanowicz 2013). In other cases, overlapping of morphometric characters was observed so that some specimens could not be unequivocally identified (Hauser 1997, Kudrna 2001, Verovnik and Glogovčan 2007). This could result from either insufficient genetic isolation of species or a greater intraspecific variation, e.g. driven by ecological factors (Bolshakov et al. 2013, Tsvetkov 2007, Fumi 2008) or from differences between the spring and summer brood (Sachanowicz 2013). Fumi (2008) also noted the potential effect of choosing poor diagnostic characters and measurement errors.

According to the discriminant criterion suggested by Fumi (2008) for females of *L. sinapis* and *L. reali*, the critical value for the ductus bursae length was 0.79 mm. According to our data, the hiatus of this character between females of *L. sinapis* and *L. juvernica* is at the interval of 0.60–0.75 mm.

The length of the saccus and valve in our case appeared insufficient for a complete discrimination of *L. sinapis* and *L. juvernica*. The ratio of these values, which allowed 98% discrimination of males of *L. sinapis* and *L. reali* (Fumi 2008), in our case also had better resolution but did not yet display a hiatus between the species (Fig. 5). Ideally, the way of discriminating should be unequivocal and not depend on geographical, ecological or seasonal circumstances. Fumi (2008) achieved 100% discrimination of males of *L. sinapis* and *L. reali* through simultaneous analysis of four genitalic characters: the lengths of the aedeagus, saccus, valve and uncus, while adding further characters to the multivariate analysis did not further contribute to resolution. However, discriminative analysis of several morphometric characters of the genitalia is laborious and hence impractical for routine identification of specimens. At present, molecular analysis involving either of our CAPS-markers is an easier means of identification a specimen than multivariate analysis of the genitalia morphology. Furthermore, the differentiation of *L. reali* and *L. juvernica* is still beyond the morphological approach and until now these species can be distinguished only by molecular markers and/or karyotype (Dincă et al. 2011, 2013).

Other external characters, such as the general size and coloration of the hind wing underside, recognised by a naked eye, are unreliable and allow only a first approach to species identification in the field (Ivonin et al. 2009), as follows from the low values of the φ coefficient for association of these characters with molecular markers. This may result from the conventional nature of the character grades or from the great variability for these characters, maybe as a remnant of introgression between species in the past. Note, however, that no males of *L. juvernica* (identified by molecular markers) were scored as '0' as to the hind wing underside suffusion (that is with well-expressed lighter spots below vein M3), the variant found in the majority of males of *L. sinapis* (Table 7). This somewhat agrees with the data by Ivonin et al (2009) who did not find external characters of *L. sinapis* among the males of *L. juvernica* identified by the genitalia.

Anyway, we conclude that molecular and karyological characters are so far the only reliable means of identification of *L. sinapis* and *L. juvernica*, and the molecular ones are much easier methodically.

Overlapping lengths of genital structures of *Leptidea sinapis* and *L. juvernica*

Divergence and fixation of alleles of genes responsible for reproductive isolation are sufficient for speciation (Wu 2001). If genitalic differences contribute to reproductive isolation, genes governing the genital structures are to occur among them quite often. Differences in the genitalia size and structure result from the realization of the ontogenetic program. Since *Leptidea sinapis* and *L. juvernica* are closely related species, they should have the same set of orthologous genes with a similar system of expression regulation in ontogenesis. A new allele in one of such 'genital' genes which once occurred in a small population can soon be fixed by gene drift, giving rise to a nascent genetically isolated species. Developmental genes often have pleiotropic effects and their mutation can bring about changes in a complex of morphological characters. In particular, the same gene may affect the length of both male and female genitalia through an effect on the size of the anlagen of genital organs in early embryogenesis in both sexes. Hence, the differences in the male and female genitalia between *L. sinapis* and *L. juvernica* may be determined by the same single major gene but also be influenced by small effects of an unknown number of other genetic and/or environmental factors. This seems to be common in *Leptidea*: thus, *Leptidea lactea* Lorkovic, 1950 was isolated from *L. morsei* because of an observed bimodal distribution of the genitalia length in united samples (Lorcovic 1950).

The overlap of distribution of the length of the male genital structures may be interpreted through presence in both species of both 'long' and 'short' alleles of the hypothetical gene responsible for differences between *L. sinapis* and *L. juvernica*, although with oppositely biased frequencies. This could result from:

- inheritance of both alleles from the common ancestor,
- introgression between species, and
- de novo mutational re-appearance of 'long' and/or 'short' alleles.

Table 7. Additional characters: the saccus curvature, general size, hind wing underside suffusion and fore wing apex shape, classified to arbitrary classes, in the studied male specimens of *Leptidea sinapis* and *L. juvernica*. Character states: saccus: 0 – straight, 1 – S-like curved; general size: 0 – large, 1 – small; hind wing underside suffusion below vein M3: 0 – with well-expressed lighter spots between veins, 1 – rather even, with very scarcely or not expressed lighter spots; fore wing apex: 0 – broadly rounded; 1 – more attenuated and acute. The typical *L. sinapis* phenotype corresponds to the character states 0000, the typical *L. juvernica* to 1111.

Specimen	Molecular identification	Saccus curvature	General size	Hind wing underside suffusion	Fore wing apex
L1	s	1	0	0	0
L2	j	1	1	1	1
L3	j	1	1	1	1
L4	s	1	0	1	1
L5	j	1	1	1	1
L6	s	0	0	0	0
L7	s	0	1	1	1
L8	j	1	1	1	0
L9	j	1	1	1	0
L10	j	1	0	1	1
L11	j	1	1	1	1
L12	j	1	1	1	0
L13	j	1	1	1	1
L14	j	1	1	1	1
L15	s	1	0	0	1
L16	s	1	1	0	1
L17	s	0	1	0	0
L18	s	0	0	0	0
L19	j	1	1	1	1
L20	j	1	1	1	1
L21	j	1	1	1	0
L22	s	1	0	0	0
L23	s	0	0	0	0
L24	j	1	1	1	1
L25	s	0	0	0	0
L26	s	1	1	1	0
L27	j	0	0	1	0
L28	j	1	0	1	0

Introgression is a common phenomenon for sympatric closely related butterfly species. According to an estimation by Mallet (2005), about 16% of 440 European butterfly species can hybridise with at least one other species in natural conditions. Most of such hybrids, especially females, suffer from lowered fertility or complete sterility in F1. However, some interspecific hybrids are still able of backcrossing with one of the parental species, which can lead to gene flow in hybrid zones (Mavárez et al. 2006, Descimon and Mallet 2009).

Specimens from Novosibirsk Province with intermediate state of diagnostic external characters or, more frequently, with discordant combination of characters of *L. sinapis* and *L. juvernica* were supposed to be interspecies hybrids or products of their backcrosses (Kosterin et al. 2007, Ivonin et al. 2009). In particular, Ivonin et al. (2009) claimed that among the spring brood males with the genitalia of *L. sinapis*, there were specimens with the external characters of *L. juvernica* (a dark, evenly suffused hind wing underside below vein M3, a processed fore wing apex). Oppositely, the males with *L. juvernica* genitalia were homogenous for these characters. Our data do not support the latter claim, for the coloration of hind wing underside and the fore wing apex shape varied strongly in males of both *L. sinapis* and *L. juvernica*.

An attempt to reveal hybridization between *L. sinapis* and *L. reali* in the Pyrenees using 16 allozyme loci was unsuccessful (Martin et al. 2003). Verovnik and Glogovčan (2007) suspected a possible hybridisation between *L. sinapis* and *L. juvernica* in Slovenia. They revealed some unusual specimens which either had the saccus of an intermediate length or had a long saccus but short aedeagus. These specimens referred to *L. sinapis* according to molecular markers. The same authors revealed two specimens morphologically corresponding to *L. juvernica* but belonging to *L. sinapis* according to the *COI* sequence. However, their RAPD analysis revealed fragments specific both to *L. sinapis* and *L. juvernica*, thus suggesting the hybrid nature of those specimens. The most recent large scale study did not detect signs of introgression among the three species of the *L. sinapis* complex (Dincă et al. 2013). On the contrary, the existence of biochemical and behavioural prezygotic barriers among them were demonstrated (Friberg et al. 2008b, Dincă et al. 2013).

Inheritance of 'long' and 'short' alleles in the common ancestor of *L. sinapis* and *L. juvernica* is also a plausible interpretation. These alleles could be involved in genetic isolation of the nascent species by forming a reproductive barrier between them, but fixation of either allele in these species may not have taken place. The initial genital prezygotic barrier could later be strengthened by adding biochemical and behavioral barriers. They would lower significance of the primary genital barrier and somewhat release selection for the 'long' versus 'short' alleles and vice versa, allowing their frequency to drift.

At this stage of our knowledge, the third scenario of arising 'long' and/or 'short' allele(s) cannot be excluded as well.

Variability of common markers versus conservation of histone H1 gene

In contrast to core histones, histone H1 is a very variable protein (Berdnikov et al. 1993, Happel and Doenecke 2009). For this reason its gene served well for reconstructing phylogeny of the genus *Pisum* L. (peas) both at inter- and intraspecies level (Zaytseva et al. 2012, 2015). In spite of its variability elsewhere, the *H1* gene appeared identical in *L. sinapis* and *L. juvernica*. The *H1* gene variation revealed in the four studied species of *Leptidea* turned out to be five times lower than that of *COI*, differing from our data obtained for three species of *Oreta* Walker, 1955 (Drepanidae) (Solovyev et al. 2015), where the substitution rate in *H1* appeared to be only twice less than that in *COI*.

Three *wsp* alleles in four *Leptidea* species

All the four studied *Leptidea* species were found to be infected with *Wolbachia*, with prevalence of infected specimens of 91.8% in *L. sinapis* + *L. juvernica* and 100% in *L. amurensis* and *L. morsei* (Table 1). We cannot exclude the possibility that all individuals in the studied populations are infected. We removed the abdomen and hence isolated DNA from somatic tissues only, while the *Wolbachia* presence can be limited to reproductive tissues (Dobson et al. 1999). The high level of infection probably indicates at functional effect of *Wolbachia* on the host, ranging from mutualism (increase of the host fitness) to a reproductive parasitism (cytoplasmic incompatibility, feminization, parthenogenesis, male-killing) (Werren 1997; Werren et al. 2008). We can exclude feminisation, male-killing or parthenogenesis that would result in biased sex ratio, which was not observed (Table 1). Special experimental studies would be necessary to investigate the reason of the high infection level in these four *Leptidea* species.

Wolbachia infection is vertically transmitted through host generations via maternal cytoplasm. Therefore the phylogeny of *Wolbachia* could be expected to be concordant with the phylogeny of its hosts. However, *Wolbachia* can as well be transmitted horizontally between related species through introgression and between unrelated species by unknown agents. In addition, *Wolbachia* strains as well as their particular genes such as *wsp* may result from recombination between different strains.

We found three *Wolbachia* strains in *Leptidea* according to the *wsp* gene sequences. Three species, *L. amurensis*, *L. sinapis* and *L. juvernica*, were found to possess allele *wsp*-10 which is widespread in insects. According to our counts at the http://pubmlst.org, it was so far registered in 27 species of Lepidoptera from different families (Pyralidae, Hesperiidae, Papilionidae, Pieridae, Nymphalidae, Lycaenidae) and, and also in *Culex pipiens* Linnaeus, 1758 from Diptera. The second allele *wsp-687* was found in *L. sinapis* for the first time. It differs from wsp-10 in only one nucleotide substitution. The third *wsp*-688 allele was also found for the first time, in *L. morsei*. This new allele has a unique hypervariable region 2, *HVR2-267*, while other hypervariable regions *HVR1-2*, *HVR3-2*, *HVR-23* were found elsewhere (Baldo et al. 2005, 2010; Baldo and Werrein 2007). Nevertheless the mentioned combination of the hypervariable regions has not been so far recorded, thus the allele is a product of recombination of strains representing different evolutionary lines of *Wolbachia*.

The pattern of *Wolbachia* variants in the studied *Leptidea* species is discordant to the host phylogeny. Variation of *wsp* sequences in *L. sinapis*, *L. juvernica* and *L. amurensis* is extremely low, viz. *wsp-10* allele is common for these species and a closely related *wsp-687* allele is also found in *L. sinapis*, whereas *L. morsei* possesses a highly divergent *wsp*-688 allele.

The *wsp-10* allele could hardly be inherited from the common ancestor of the three species, taking into account a considerable degree of variation accumulated by the host *Leptidea* genes, both nuclear and mitochondrial (Dincă et al. 2011, 2013 and this paper). Note that supposition (i) contradicts also the phylogenetic relationships of the species involved (Dincă et al. 2011) as follows: the branch *L. morsei* + *L. amurensis*

is opposed to the branch containing *L. sinapis* and *L. juvernica*. The low *wsp* variation in three species may have two explanations:

(i) the same strain of *Wolbachia* could have spread across the three species by interspecies crosses;
(ii) The same *wsp* allele could have spread across the three species via horizontal transfer of *Wolbachia*.

We exclude option (i) since we failed to trace such crosses by other molecular means. Explanation (ii), that is independent infection by the same *Wolbachia* strain, is most probable because of a high frequency of *wsp-10* in butterflies. *L. morsei* was no doubt independently infected by an unusual *Wolbachia* strain with *wsp*-688, however, more data on *L. morsei* is necessary to consider the evolutionary history of its *Wolbachia*.

Conclusions

Leptidea amurensis, *L. morsei*, *L. sinapis* and *L. juvernica* coexist in the same locality in West Siberia without detectable introgression. Each of the molecular characters *COI*, *CAD* and *ITS2* markers, as well as the length of the female ductus bursae, allow a reliable identification of *L. sinapis* and *L. juvernica*. The length of the saccus related to that of the valva as the most easily assessed male genitalic character, as well as the characters of wing pattern and shape in males, are unreliable for identification of these two species. An overwhelming majority of *Leptidea* individuals are infected with *Wolbachia*. Three alleles of the *Wolbachia* gene *wsp* were recorded (two of them for the first time), that of *L. morsei* being highly divergent from the allele found in *L. amurensis*, *L. juvernica* and *L. sinapis* (this species contains a very similar third allele), which is discordant with the presumed phylogeny of the host.

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